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A ROLE FOR THE AUTONOMIC NERVOUS SYSTEM IN MODULATING THE IMMUNE RESPONSE DURING MILD EMOTIONAL STIMULI.

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SUMMARY

The role of the autonomic nervous system in the modulation of the immune response to emotional stimuli, was established in rats subjected to the passive avoidance test. An increase in splenic primary antibody response directed against SRBC was found after exposure of rats to the passive avoidance apparatus (novelty). Both local surgical denervation of the spleen and ß-receptor blockade (timolol, 1 mg/kg i.p. 1 h prior to testing) prevented the increase in primary antibody response.

The presence of an anatomical link between the brain and the immune system argues strongly for a role of the autonomic nervous system in translating central neural processes into signals that can influence cells of the immune system (6, 13, 14, 25). The spleen postganglionic sympathetic axons, arising from the superior mesenteric-coeliac ganglion were shown to associate not only with blood vessels but also with lymphocytes and macrophages (13). Norepinephrine (NE) containing fibres were demonstrated in T cell areas, mainly in the parenchyma of the periarteriolar lymphoid sheath (PALS). The B cell areas (the follicles) are less densely innervated with only some delicate nerve fibres ending adjacent to both T and B lymphocytes (1, 13). The actual release of NE was demonstrated by *in vivo* microdialysis techniques (16). Immunoregulation is further conceived to be mediated by the sympatho-adrenal system as suggested by the demonstration of α- and ß-adrenoceptors on lymphocytes (11, 18).

Catecholamines were shown to be involved in the generation and maturation of lymphocytes and in antibody formation (for review see 14). *In vitro* they can influence intracellular cyclic nucleotide levels, lymphoid cell proliferation, antibody formation and IL-2 receptor expression etc. (21, 23, 7, 12).

In the present study the role of the autonomic nervous system was studied in immunomodulation observed after exposure of rats to different environmental stimuli. The passive avoidance test was chosen as a model for subjecting rats to emotional stress, such as exposure to a novelty and to a conflict situation (2). The passive avoidance test is based on the innate preference of rats for the dark. After habituation to the apparatus rats receive

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a short-lasting mild electric footshock in the dark cage (learning trial). 24 h later, when the rat is tested for retention, it will consequently avoid the dark cage (passive avoidance behaviour). Subjecting rats to the passive avoidance test has definite neuroendocrine consequences (f.i. elevation of plasma corticosterone level (8). In previous studies we have demonstrated that the immune system can be modulated in a situation-specific manner; exposure of rats only to the passive avoidance apparatus (novelty) resulted in an increase of the anti-Sheep Red Blood Cell primary antibody response. When rats are tested for retention in a passive avoidance test (conflict situation) there is not only a blockade of the stimulation but an actual decrease in immunological response relative to baseline homecage controls (8, 9). The two different situations resulting respectively in increase and a blockade of the increase (suppression) of the immune response prompted us to investigate the role of the autonomic nervous system in the pattern of immunomodulation. To that end the sympathetic nervous system was temporarily blocked by the ß1ß2 blocker timolol. Permanent blockade of the autonomic nervous system was effectuated by surgical denervation of the afferent and efferent nerves from the spleen.

MATERIAL AND METHODS

Animals

Male inbred Wistar rats of 140-180 g (± 7 weeks old) were used (bred in our laboratory). One day before the start of the passive avoidance test rats were moved to low stress animal facilities, next to the experimental room. These animal facilities were created to satisfy the experimental needs for the study on stress and immunology: a sound-attenuated room, no motion of cages and no housing of other animals. The cages were not cleaned during the course of the experiment.

Step through type one-trial-learning passive avoidance conditioning (2)

This test uses the innate preference of rats for a dark environment. The experimental apparatus consists of a dark compartment equipped with a grid floor, to which an elevated and illuminated platform is attached. During passive avoidance conditioning the illuminated platform is the only light source in the room. On day 1, after habituation to the dark compartment for 2 minutes, the rat was placed on the illuminated platform and allowed to enter the dark box (training trial). Upon entry the door was closed and the animal remained in the box for 10 seconds and the rat was subsequently placed in its home cage. Three such trials were run on day 2. During the third trial (learning trial; LT) the rat received a single scrambled electric footshock for 2 seconds through the metal grid floor of the cage immediately upon entering the dark compartment. The shock intensity applied was 0.5 mA. Retention (RT) was tested 1 day after the learning trial by measuring the entrance latency up to a maximum of 300 sec. With the shock intensity applied (0.5 mA) 90% of all rats showed maximal avoidance behaviour (300 sec.) Immediately after the retention test rats were immunized. Rats that received a learning trial and that were subsequently tested for retention, will be referred to as 0.5 mA + RT. The apparatus control group did not receive a footshock (0 mA + RT)(entrance latency 3-7 sec). Home-cage control animals (HCC) were not subjected to the passive avoidance test, but were only handled for 2 minutes per day, the same day the other rats were trained in the passive avoidance test.

All experiments were carried out between 8:00 A:M: and 11:00 A:M:.

Immunization and measurement of the number of plaque-forming cells (PFC's)

Rats were immunized i.p. with 10^9 SRBC in a volume of 0.5 ml saline. The spleen was removed under ether anaesthesia 5 days after immunization and was minced through a small-mesh wire netting. The cell suspension thus obtained was washed twice in minimum essential medium (MEM; Gibco, Europe), supplemented with 100 IU/ml penicillin, 0.01% streptomycin and 0,01% bovine serum albumin (BSA; Organon Technika, the Netherlands). Lymphocytes were adjusted to a concentration of 2.5 x 10^6 cells/ml in MEM 1% BSA.

Subsequently 20 µl of the cell suspension was placed on a monolayer of SRBC together with 20 µl guinea pig complement (1:5). The number of IgM plaque-forming cells (PFC) was determined in a PFC test as described by Heijnen *et al.* (19).

Surgical denervation

Rats were anaesthetized with Hypnorm (Janssen Pharmaceuticals, Beerse, Belgium). The spleen was denervated in the proximity of the spleen (See Discussion). Rats were allowed to recover for 5 days. Eight days after denervation rats were immunized. Denervation of the spleen was checked in a separate group of animals by assessment of catecholamine level in the spleen 8 days after denervation at the time rats of the other groups were immunized.

Determination of Catecholamine Levels

The spleens were homogenized in iced 0.2 N perchloric acid using a ground glass homogenizer to extract the catecholamines and precipitate the proteins. After centrifugation (15000 x g for 15 min at 4°C), 20 µl of the supernatants were analyzed radioenzymatically for norepinephrine (NE) and epinephrine (E) according to Van der Gugten et al. (24). The catecholamines were converted into their (^{3}H)-methoxy derivatives by incubation with S-adenosyl-L-(methyl-^{3}H)methionine (60-80 Ci/mmol; NEN chemicals) in the presence of catechol-O-methyltransferase. Labeled products were isolated by organic extraction and paper chromatography. After elution of the labeled products, activity was counted in a liquid scintillation analyzer. CA concentrations were expressed as ng/g wet tissue. This assay has an intra-assay variability of less than 10%. It is sensitive to 2 pg for NE and 1 pg for E. Samples from one experiment were run in the same assay.

Effects of surgical splenic denervation on the PFC response of rats subjected to the passive avoidance test

Rats underwent a splenic denervation (den) or sham-denervation. Five days after the operation rats were divided into a HCC group (sham n=6; den n=7), a 0 mA + RT (sham n=7; den n=7) and a 0.5 mA + RT group (sham n=7; den n=7). On day 3 of the passive avoidance test all rats were immunized immediately after the retention test. Rats of the HCC group were also immunized at the same day. Five days after immunization the PFC response was determined.

Effects of chemical splenic denervation of the PFC response rats subjected to the passive avoidance test

Rats were divided into a HCC (sham n= 5, timolol n=5), a 0mA + RT (sham n=10, timolol n=10, and a 0.5 mA + RT group (sham n=9; timolol n=9).
One hour prior to the retention test rats received 1 mg/kg timolol i.p. Immediately after the retention test rats were immunized. Rats of the HCC group were immunized 1 h after timolol administration. Five days after immunization the PFC response was determined.

Statistics

Avoidance latencies were expressed as the median of the group. The PFC's are expressed as mean ± SEM. For statistical evaluation of these results a two-way analysis of variance was used. The Student's t-test was used to compare group means. In all tests $p < 0.05$ was taken as the level of significance.

RESULTS

Effects of surgical splenic denervation on the PFC response of rats subjected to the passive avoidance test

In rats, that had remained in their home cages (HCC) no effect of surgical denervation of the spleen on the PFC response was observed (see Fig. 1). In sham-denervated rats

exposure to the apparatus (0 mA + RT) resulted in a stimulation of the PFC response as compared with the response of the sham-denervated rats that had remained in their home cage (HCC). In the 0 mA + RT group the number of PFC's of rats with denervated spleens was significantly lower than the number of PFC's of rats with sham-denervated spleens of the concomitant group (Student's t-test; $p < 0.05$). Apparently denervation blocked the increase in PFC response in the 0 mA + RT group.

However, surgical denervation did not influence the PFC response of rats that showed passive avoidance behaviour; no difference in number of PFC's was observed between denervated rats and sham-denervated rats that showed passive avoidance behaviour (median 300 sec.) of the 0.5 mA + RT group. The number of PFC's of rats with denervated or sham-denervated spleens of the 0.5 mA + RT was significantly lower (Student's t-test; $p < 0.05$) than that of rats of the sham-denervated apparatus control group (0 mA + RT) and of the sham-denervated rats of the HCC group (see Fig. 1).

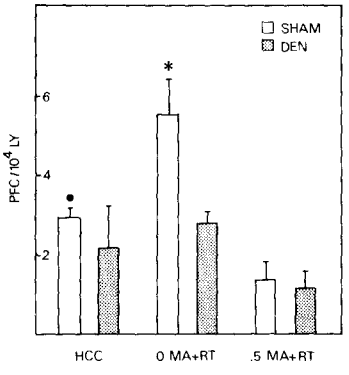

Fig 1.

Effects of surgical splenic denervation on the PFC response of rats of the HCC, 0 mA + RT and the 0.5 mA+RT group. Results of a two-way ANOVA: F (shock): 40 (2,40) $p < 0.001$. F(denervation): 17 (1,40) $p < 0.001$. F (shock x denervation): 17 (2,40) $p < 0.001$. The number of PFC's of the splenic denervated rats of the 0 mA + RT group was lower than that of the sham denervated rats of the 0 mA + RT group (* $p < 0.05$; Student's t-test).

The number of PFC's of sham and splenic denervated rats of the 0.5 mA + RT is lower than that of sham-denervated rats of the HCC group (o $p < 0.05$; Student's t-test).

Effects of ß-receptor blockade on the PFC response of rats subjected to the passive avoidance test

Intraperitoneal administration of Timolol (1 mg/kg) did not affect the primary antibody response of rats that remained in their home cages (HCC group; see Fig. 2).

A stimulation of the PFC response was observed in the saline pretreated rats of the apparatus control group (0 mA + RT) as compared with the response of rats that had remained in their home cage (HCC). In the 0 mA + RT group the number of PFC's of rats pretreated with timolol was significantly lower than the number of PFC's of rats of the placebo treated group.

The number of PFC's of timolol pretreated and placebo pretreated rats of the 0.5 mA + RT group, that showed avoidance behaviour (median avoidance latency: 300 sec.) was significantly lower (Student's t-test; $p < 0.05$) than the number of PFC's of the placebo pretreated rats of the 0 mA + RT group. No difference in PFC response was observed between timolol and placebo pretreated rats of the 0.5 mA + RT group.

Splenic NE and E level

At the time of immunization a separate group of rats was sacrificed under ether anaesthesia to determine the splenic NE and E level. In sham-denervated rats ($n = 5$) the NE level was 712 ± 168 ng/g splenic tissue and the E level was 17 ± 3 ng/g splenic tissue. In rats with surgical denervated spleens ($n = 5$) the NE level was 38 ± 11 ng/g splenic tissue and the E level was 9 ± 3 ng/g splenic tissue.

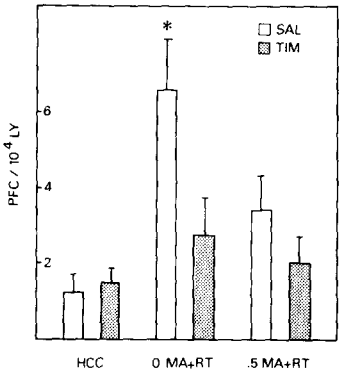

Fig 2.

Effects of timolol (1 mg/kg i.p.) 1 h prior to immunization on the PFC response of rats of the HCC, 0 mA +RT and the 0.5 mA + RT groups. Results of a two-way ANOVA: F (shock): 7.4 (2,47) $p < 0.005$. F (timolol): 10 (1,47) $p < 0.005$. F (shock x timolol): 2 (2,47) not significant. The number of PFC's of timolol treated rats of the 0 mA + RT group was lower than that of the saline treated rats of the 0 mA + RT group (* $p < 0.05$; Student's t-test).

DISCUSSION

The results of this study favour a role for the sympatho-adrenal system in the stimulation of the splenic anti-Sheep Red Blood Cell primary antibody response induced by mild environmental stimuli. In rats that were subjected to a novel environment (apparatus control group) an increase in number of PFC's was observed. Surgical denervation of the spleen could effectively block the increase in primary antibody response. The involvement of the sympatho-adrenal system in immunomodulation was further supported by pharmacological blockade. In this study the β_1/β_2 adrenoceptor blocker timolol was used. Both measures to block autonomic c.q. sympathetic activity prevented the increase in the primary antibody response in non-shocked rats (0 mA + RT group). Apparently environmental stimuli, such as exposure to the apparatus, are perceived as a mild stimulus by the CNS and resulted in a stimulation of the peripheral sympathetic nerve innervating the spleen, leading to an enhanced PFC response.

Other physiological systems like blood pressure and heart rate are also under the control of the autonomic nervous system. In this respect it is of interest that in non-shocked rats (0 mA + RT) a tachycardiac response was observed, suggesting an increase in sympa-

thetic activity (Dr. W. Ruigian, personal communication). Our observations are supported by the data of Fujiwara et al. (16) who found an increase in anti-SRBC PFC's after subjecting mice to mild stressful stimuli as Haffner's stimuli or physical stimuli such as hair plucking. The increase in PFC's was ascribed to the ß-action of endogenous catecholamines; the ß-blocker propanol blocked the increase of the PFC response, whereas the α-blocker phentolamine was not active in this respect.

In a previous study we have demonstrated an increase in PFC response in rats that had received a learning trial (0.5 mA), but that were not tested for retention (10). When rats of the 0.5 mA + RT were tested for retention, they showed avoidance latencies of 300 sec.. In these rats a pronounced increase in plasma corticosterone level was observed (8). This experimental group was also incorporated in the present study. In these rats (sham operated, or saline pretreated of the 0.5 mA + RT group), the initially enhanced PFC response did not occur (Fig. 2) or was even decreased (Fig. 1). Apparently the immunomodulation in the passive avoidance model may be ascribed to two different mechanisms: one leading to stimulation (in the 0 mA + RT group) and another responsible for blocking the stimulation of the response (in the 0.5 mA + RT group).

It is of interest to mention that during passive avoidance behaviour a bradycardiac response was observed, suggesting an increase in parasympathetic activity (5). The pattern of immunomodulation in this study shows a similarity with the changes in heart rate as reported by Bohus (5). Thus the blockade of the stimulation of the primary antibody response may also be the consequence of increased parasympathetic activity during passive avoidance behaviour (in the 0.5 mA + RT group). However a suppressive activity can not only be ascribed to the parasympathetic nervous system, since a decrease in primary antibody response was also observed in rats of the 0.5 mA + RT group with a surgically denervated spleen. Therefore it is highly likely that circulating hormones, such as corticosteroids play a role in the down-regulation of the PFC response; in rats tested for retention (0.5 mA + RT group) a significant increase in plasma corticosteroid level was observed (8, 9).

Histological studies in the spleen showed nerve endings in a T lymphocyte and monocyte rich area (PALS) (14). We therefore assume that the autonomic nervous system may affect the primary antibody response mainly by modulating the activity of T cells and monocytes. This idea is supported by the finding that mitogen induced T cell proliferative responses of non-shocked control rats (0 mA + RT group) is affected in a similar way as the primary antibody response (8, 9).

The present study emphasizes the importance to define the basal state of activity of the autonomic nervous system, c.q. the state of arousal at the time of immunization for measuring the effect of environmental stimuli on the immune system. Under controlled experimental conditions changes in environmental stimuli, such as exposure to a novel situation and mild stress are perceived by the CNS and conveyed to the immune system in the spleen, via the sympathetic nervous system.

In the present experiment denervation per se did not result in an altered PFC response; no effect of surgical or sympathetic blockade was observed in rats of the home cage control group. This indicates that neither the autonomic innervation nor ß-adrenergic mechanisms play a role in the basal state of activity of the lymphocytes.

In many studies a reduction of the PFC response to T cell dependent antigens was observed after pharmacological sympathetic blockade (14, 21). This may depend on the state of arousal of the animal (state of the autonomic nervous system) which may lead to an increased immune response. In other studies no alteration in PFC response after similar blockade was observed (23).

In conclusion, this study suggests a role for the sympatho-adrenal system in stimulating the primary antibody response induced by subjection of rats to the passive avoidance apparatus.

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